Bioimmunological activities of *Candida glabrata* cellular mannan

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Abstract

Candida glabrata is a second most common human opportunistic pathogen which causes superficial but also life-threatening systemic candidiasis. According to the localization of mannans and mannoproteins in the outermost layer of the cell wall, mannan detection could be one of the first steps in the cell recognition of *Candida* cells by the host innate immune system. Mannans from the cell wall provide important immunomodulatory activities, compromising stimulation of cytokine production, induction of dendritic cells maturation and T-cell immunity. The model of DCs represents a promising tool to study immunomodulatory interventions throughout the vaccine development. Activated DCs induce, activate and polarize T-cell responses by expression of distinct maturation markers and cytokines regulating the adaptive immune responses. In addition, they are uniquely adept at decoding the fungus-associated information and translate it in qualitatively different T helper responses. We find out, that *C. glabrata* mannan is able to induce proliferation of splenocytes and to increase the production of TNF-α and IL-4. Next, increased the expression of co-stimulatory molecules CD80 and CD86 and the proportion of $CD4^+CD25^+$ and $CD4^+CD28^+$ T cells during *in vitro* stimulation of splenocytes.

1. Introduction

Several opportunistic fungal pathogens such as *Aspergillus, Candida, Cryptococcus* are frequently distributed and represent ethiological agents of majority of invasive fungal infections.

Generally, Candida glabrata is a part of the mycobiota of mucosal surfaces and gastrointestinal tract of healthy individuals (Hallen-Adams & Suhr, 2017). *C. glabrata* is non-dimorphic yeast, contrary to *Candida albicans* and other pathogenic *Candida* species; frequently found in a blastoconidia form

as a commensal but can easily turn as a pathogen (Fidel*, et al.*, 1999)., *C. glabrata* has been long considered as non-pathogenic, causing life-threatening infections in humans only rarely (Fidel*, et al.*, 1999). In last decade, the frequency of mucosal and systemic infections caused by *C. glabrata* has significantly increased because of increasing use of broad-spectrum antimycotics together with immunosuppressive therapy (Fidel*, et al.*, 1999). Depending on the site of infection, *C. glabrata* occurred as the second or the third most common cause of candidiasis after *C.albicans and C.parapsilosis* (Pfaller*, et al.*, 2011). Various cellular and molecular defects are the predisposition factors in candidosis e.g. defects of the dectin-1/CARD9-MALT1-BCL10 signaling pathway are associated with chronic mucocutaneous candidiasis,and defects of the IL-12/IFN-γ pathway and T-helper 17-mediated response are associated with increased susceptibility to endemic mycoses (Vinh, 2011, Lanternier*, et al.*, 2013, Lee & Lau, 2017). *C. glabrata* infections have a high morbidity and mortality rate in immunocompromised persons. Candida glabrata is the second most common pathogen of severe candidiasis in immunocompromised hosts, following C. albicans (Chen*, et al.*, 2017).) *C. glabrata* clinical isolates are highly resistant to azole antifungal agents, especially fluconazole in 15%-25% of cases and has decreased susceptibility to most antifungals,(Fidel*, et al.*, 1999) with high capacity to produce biofilms (Rodrigues*, et al.*, 2014),

The host immune response to the fungal infection depends on the antigenic determinants recognition and on effector cells activation. The majority of fungi are detected and identified by cells of innate immune system (Herring & Huffnagle, 2001, Romani, 2004).

Anti-fungal adaptive immunity is induced by antigen presenting cells, most importantly by dendritic cells (DCs), currently believed to be the conductors of this complex cooperation. DCs activate T cells through expression of co-stimulatory molecules and trigger release of cytokines being able to regulate the adaptive immune response (Colonna*, et al.*, 2006). Inflammatory DCs initiate antifungal Th17 and Th2 cell responses, while tolerogenic DCs activate Th1 and Tregs. The balance between these two subsets of DCs is determined, in part, by the kynurenine pathway and tryptophan catabolism, and involves indoleamine 2,3-dioxygenase (Romani, 2011).

Although *C. glabrata* lacks several virulence factors of *C. albicans*, such as hyphae formation and secretion of proteinases, and differs in key aspects of host – fungus interactions from *C. albicans*, successfully represents second most common cause of systemic candidiasis. Innate immune cells such as dendritic cells and macrophages establish the first line of defence against microbial pathogens (Brown, 2011, Cheng*, et al.*, 2012). Dendritic cells recognize and phagocytose *C. albicans* to process them for antigen presentation and differentiate between yeast and hyphal morphoform, thus initiate the T helper cellular immune response, essential requirement for long term resistance to

candidiasis (d'Ostiani*, et al.*, 2000, Newman & Holly, 2001). It is established that interactions between different innate immune cells receptors and components of the fungal cell wall trigger phagocytosis and subsequent immune responses (Netea*, et al.*, 2008, Kiyoura & Tamai, 2015), but the ligands mediating the appropriate host immune response have not been identified until yet. It was described that activation of host immune responses occurs after the initial recognition of mannan or a mannosylated protein by murine macrophages (Keppler-Ross*, et al.*, 2010). Moreover, published results pointed out preference by J774 macrophages, in which *C. glabrata* yeast cells are preferred over *C. albicans* yeast cells suggesting mannose or some mannosylated protein as a critical recognition ligand (Keppler-Ross*, et al.*, 2010).

Yeasts, including *C. glabrata*, were shown to effectively induce the expression of the activation marker CD83, the co-stimulatory molecules CD80, CD86, CD54, CD58, and CD40, as well as the antigen-presenting molecules MHC I and MHC II on DCs (Bazan*, et al.*, 2018). Efficient control of *Candida* relies on the generation of a protective Th1 response that is initiated by IL-12 secreted by DCs and phagocytic cells. IL-12 induces T cells to produce IFN-γ, a key activator of effector cells (neutrophils and macrophages) and ensures prolonged responsiveness of $CD4^+$ Th1 cells to IL-12 stimulation. It has been reported that yeast and hyphae morphoforms lead to differential activation of DCs, where hyphae inhibit IL-12 production, while inducing IL-4 (d'Ostiani*, et al.*, 2000). *In vitro* studies have shown that both human and murine DCs recognize and internalize *Candida* cells (d'Ostiani*, et al.*, 2000, Bacci*, et al.*, 2002, Bozza*, et al.*, 2004, Romagnoli*, et al.*, 2004, Torosantucci*, et al.*, 2004) and that fungi and fungal products may affect DCs functioning as well (Romagnoli*, et al.*, 2004, Torosantucci*, et al.*, 2004). It was described, that yeast-activated DCs secrete various cytokines including inflammatory TNF-α, IL-6, IL-8, and IL-1β or T-cell polarizing IL-12, IL-10, IL-23, and IL-27 (Bazan*, et al.*, 2018).

Mannan polysaccharide is exposed at the most external layer of the cell wall and is involved in several types of interactions of fungal cells with host immune system. Mannan consists of backbone with α-1,6-bonds and mannoside side chains of varying length, containing α-1,2, α-1,3 and β-1,2 bound types (Kobayashi*, et al.*, 1992, Shibata*, et al.*, 2007). The mannans of medically important *Candida* species contain different structures that correspond to the species-specific antigens(Suzuki, 1997a, Suzuki, 1997b). α-Linked mannose residues are known to be involved in host–pathogen interactions due to the interaction with mannose receptor, mannan-binding protein, and Dectin-2 (Ifrim*, et al.*, 2016) as well as the dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN) (Cambi*, et al.*, 2008, Netea*, et al.*, 2008). Structural studies indicate that *C. glabrata* mannan is more closely related to *S. cerevisiae* mannan with the absence of long side chains and 3,6-branched mannose residue in the side chains of the mannan, present in *C. albicans* mannan

(Takahashi*, et al.*, 2012). Moreover, *C. glabrata* mannan shows some inter-strain variations (Takahashi*, et al.*, 2012).

2. **Material and methods**

2.1 Mannan preparation

Yeast strains *C. glabrata* CCY 26-20-1, *C. albicans* CCY 29-3-32, and *S. cerevisiae* CCY 21-4-13 (Culture Collection of Yeast, Institute of Chemistry of Slovak Academy of Science, Bratislava, Slovakia) was cultured on semi-synthetic 2 % glucose medium at 28°C for 4 days. Cellular mannans was extracted by autoclaving in 0.2 mol/l NaCl (120°C, 700 kPa) for 10 minutes and purified using precipitation with Fehling's reagent (Peat*, et al.*, 1961). The mannans were analyzed for carbon, hydrogen, and nitrogen content using the EA 1108 device (FISONS Instruments, UK). Mannans´ stock solution and individual exposition doses were prepared aseptically using apyrogenic, sterile aqua pro injectione (Fresenius, Kabi Italia S.r.l., Verona) under sterile laminar flow conditions (Biohazard II, Esco). The stock solution was sterile filtered using a syringe with a 0.2-µm filter (Q-Max®Syringe filter) and was assayed with an EndoLISA® ELISA-based Endotoxin Detection Assay (Hyglos) and evaluated with a Cytation 5 Imager Multi-Mode Reader (BioTek, USA). The average value of three individual measurements was 0.01 EU ml/1, confirming an endotoxin-free solution.

2.2 NMR spectroscopy of mannan

C. glabrata mannan sample was exchanged twice with D_2O, lyophilized between the exchanges and then dissolved in 600 µL of 99.95% D_2O. 1 µl of acetone was added as internal standard (2.225/31.07 ppm). NMR spectra were recorded on Bruker Avance III HD 600 MHz spectrometer equipped with inverse liquid nitrogen cooled cryoprobe (Prodigy) at 45°C. 1D ^{1}H, and ^{1}H-^{13}C perfect heteronuclear HSQC spectra were acquired and processed using Bruker TOPSPIN software. Perfect HSQC is special case of HSQC pulse sequence where the spectrum is acquired with homonuclear "decoupling" so its lineshape is pure positive phase and is quantitative (Castanar*, et al.*, 2015). ^{1}H NMR and ^{1}H–^{1}H 2D TOCSY spectra were acquired in D_2O (99.97 % D) at 45 °C on a Bruker AVANCE III HDX 600 MHz spectrometer (Bruker BioSpin, Rheinstetten, Germany) equipped with a triple inverse TCI H-C/N-D-05-Z liquid He-cooled cryoprobe and processed using MestReNova 12.0.3 software. The ^{1}H signal of acetone (2.225 ppm) was used as a reference for chemical shifts.

2.3 Experimental animals

CD1 mice (female, 4 - 6 weeks old, purchased from Charles River Breeding Laboratories, Calco, Lecco, Italy) were used for isolation of DCs and subsequent analysis of DCs activation and co-stimulation with T cells. BALB/c mice (female, 6 weeks old, obtained from Research Institute of

Animal Production Velaz, Prague, Czech Republic) were used for isolation of splenocytes and following analyses of cytokine production and proliferation assay.

2.4 Activation of splenocytes

Female Balb/c mice aged 8–12 weeks were obtained from the breeding facility VELAZ, Prague (Czech Republic). *Ex vivo* animal experiments were conducted in compliance with GLP and OECD guidelines, according to the ethical guidelines issued by the Research Base of Slovak Medical University, Institute of Preventive and Clinical Medicine (Bratislava, Slovakia), under the approval of State veterinary and food administration of the Slovak Republic. The animals were provided food and water ad libitum and maintained in hoods under laminar flow conditions. Aseptically removed spleens were placed into sterile ice-cold saline (1 ml per spleen) and homogenized with the plunger end of the syringe. The cell suspension was centrifuged at 800 × g for 10 min at 4°C. The cell pellet was resuspended in 5 ml of ACK lysis buffer (0.15 M NH_4Cl, 1 M K_2CO_3, and 0.01 M EDTA, pH 7.2) and incubated at room temperature for 5 min to lyse the red blood cells. The cell suspension was washed twice with saline and resuspended in complete RPMI-1640 (Lonza, Belgium) containing 10 % fetal bovine serum, 100 U/ml penicillin and 100 mg/ml streptomycin sulphate (Gibco, Germany). The cell density was adjusted to 1×10^6 cells per ml with RPMI-1640 following the determination of cell viability using trypan blue dye exclusion method. Splenocytes were seeded into 24 well culture dishes (suspension containing 4×10^5 cells per well) and stimulated *in vitro* with LPS (final concentration 10 µg/ml, Sigma) and *C. glabrata* mannan (400 µg/ml and 800 µg/ml) for 24 h at 37 °C in a humidified incubator with a 5% CO_2 atmosphere. After stimulation the proliferation of spleen cells and ELISpot analyses of TNF-α production were performed and culture media were stored at -20°C for determination of cytokines.

2.5 Cell culture and exposition

Cell line murine macrophages RAW 264.7 (ATCC®TIB-71™, ATCC, UK) were cultured in complete Dulbecco's Modified Eagle Medium for 24 h at 37°C in an atmosphere of 5% CO_2 and 100% relative humidity until approx 80% confluency. Cell viability has been assayed with the Trypan blue dye using a TC20™ automated cell counter (Bio-Rad Laboratories, Inc., USA). The starting inocula of 1×10^5 cells/mL/well (approx 90% of viable cells) were seeded onto a 24-well cell culture plate (Sigma-Aldrich, USA) and treated with 10 and 100 µg per well of mannan formulas, respectively. Cell mitogens Concanavalin A (Con A, 10 µg/mL, Sigma-Aldrich, St Louis, MO, USA), phytohemagglutinin (PHA, 10 µg/mL, Sigma- Aldrich, St Louis, MO, USA), pokeweed mitogen (PWM, 1 µg/ mL, Sigma-Aldrich, St Louis, MO, USA), and *E.coli* lipopolysaccharide (LPS 10 µg/mL,Sigma- Aldrich, St Louis, MO, USA) were used as positive controls. Untreated cells were used as negative control. *In vitro*

exposition was performed for 24, and 48 h, respectively. The exposed cells were subjected to microscopic observation of morphological characteristics (Zoe™ fluorescent cell imager, Bio-Rad). The cell culture media were stored at −20°C until further use.

2.6 ELISPOT analysis of TNF-α production

Splenocytes were seeded in concentration 5×10^4 cells per well for the direct production and further enumeration of TNF-α producing cells onto anti-mouse TNF-α antibody pre-coated PVDF microplate. Following steps and procedures were according to manufacturer's recommendation (Mouse TNF-α ELISPOT, BenderMedSystems, Austria). Quantitative evaluation of spots was performed via KS ELISPOT 4.10 running under AxioVision software using Imager A.1 Microscope (Zeiss, Germany).

2.7 Proliferation

Proliferation of exposed splenocytes and RAW 264.7 cells was evaluated on the basis of ATP concentration measured by ViaLight™ plus kit (Lonza, Walkersville, Maryland, United States), using a microtitre plate computer-driven luminometer Immunotech LM-01T (Immunotech, Prague, Czech Republic). In this method, ATP was assessed enzymatically with firefly luciferase which catalyses the hydrolysis of ATP and oxidation of D-luciferin. As ATP is the limiting reagent in this reaction, the light reaching the photomultiplier tube of the luminometer is proportional to the amount of ATP. Light emission, expressed as relative light units (Mazzolla*, et al.*), was recorded continuously for 180 s and evaluated based on integral (peak) values. (Light emission, recorded during 1 s reading, and was expressed in relative light units - RLU).

2.8 ELISA determination of cytokines

The concentration of interleukins and growth factors in cell culture media supernates following the exposure with mannans were assayed with Platinum ELISAs®: Mouse IL-12 (p70) (Cat#BMS616, (minimum detectable dose) MDD 4 pg/mL), Mouse GM-CSF (Cat#BMS612, MDD 2 pg/mL), Mouse IL-17(Cat#BMS6001, MDD 1.6 pg/mL), and Mouse IL-6 (Cat#BMS603/2, MDD 6.5 pg/mL); Instant ELISAs®: Mouse IL-1β (Cat#BMS600/2INST, MDD 3 pg/mL), Mouse tumor necrosis factor (TNF)-α (Cat#BMS607/2INST, MDD 4 pg/mL), Mouse IL-10 (Cat#BMS614INST, MDD 5.28 pg/mL), Mouse IL-4 (Cat#BMS613INST, MDD 0.6 pg/mL) and Mouse IFNγ (Cat#BMS606INST, MDD 4.0 pg/mL),,all from Affymetrix e-Bioscience, USA, according to the instructions and test protocols of the manufacturer.

2.9 $CD11c^+$ DCs separation

$CD11c^+$ DCs were separated from spleens of CD1 mice using CD11c (N418) mAbs - conjugated MicroBeads (Miltenyi Biotec, Bergisch Gladbach, Germany), followed by magnetic separation

according to the manufacturer's instructions. After magnetic separation, $CD11c^-$ cells were harvested, washed with RPMI 1640 medium adjusted with 10% FBS, 2mM L-glutamne, 100U/ml penicillin and 100 µg/ml streptomycin (compete RPMI 1640 medium) and incubated overnight in 48-well flat-bottom culture plates.

2.10 DCs stimulation and flow cytometry analysis

For analysis of CD80 and CD86 time related expression, purified splenic $CD11c^+$ DCs from CD1 mice (5×10^5 cells per ml) were stimulated for 24 and 48 h with 10 µg/ml *Escherichia coli* lipopolysaccharide (LPS) and 100 µg/ml of *C. glabrata* mannan. For concentration dependent stimulation purified splenic $CD11c^+$ DCs from CD1 mice (5×10^5 cells/ml) were stimulated for 24 h with 10 µg/ml *E. coli* LPS and *C. glabrata* mannan in concentrations: 100µg/ml, 300µg/ml and 600µg/ml. After stimulation, cells were harvested and incubated for 30 min at 4°C with fluorochrome conjugated monoclonal antibodies Anti-Mouse CD80-PE (clone YTS, Rat IgG2b), Anti-Mouse CD86-PE (clone YTS, Rat IgG2b), Anti-Rat IgG2a-FITC (clone YTS, Rat IgG2b), Anti-Rat IgG2b-PE (clone YTS, Rat IgG2b), (Antigenix America Inc., USA). After incubation, cells were analyzed using a FACScan flow cytofluorometer (BD Biosciences, Franklin Lakes, NJ). Control staining of cells with irrelevant antibodies was used to obtain background fluorescence values. Data are expressed as mean percentage of $CD80^+$ DCs and mean percentage of $CD86^+$ DCs out of $CD11c^+$ DCs ± SD and the mean fluorescence intensity (MFI) of $CD80^+$ DCs and $CD86^+$ DCs. The analysis was performed using Kaluza Flow Cytometry Analysis 1.0 software (Beckman Coulter Inc., Fullerton, CA, USA) (Supplementary Fig. S1, Supporting Information).

2.11 Lymphocytes co-stimulation with prestimulated DCs and flow cytometry analysis

Purified splenic $CD11c^+$ DCs from CD1 mice (5×10^5 cells per ml) were pulsed with *E. coli* LPS (10 µg/ml) and mannan *C. glabrata* (100 µg/ml) for 24 h at 37°C in a humid atmosphere of 5% CO_2. *C. glabrata* mannan, LPS pulsed and unstimulated DCs were collected, washed and resuspended at 5×10^5 cells per ml with complete RPMI 1640 medium. CD11c depleted splenocytes were maintained for 24 h at 37°C in a humid atmosphere of 5% CO_2. Thereafter, non-adherent cells were harvested from CD11c depleted splenocytes, washed and adjusted to 5×10^6 cells per ml in complete RPMI 1640 medium. Autologous co-cultures were performed in 48-well plates containing pulsed or control unstimulated DCs (1×10^5 cells per well) and non-adherent spleen cells (1×10^6 cells per well) and DCs (1×10^5 cells per well) in complete RPMI 1640 medium at a final volume of 500 µl per well. Co-cultures were incubated for 48 h or 5 days at 37°C in a humid atmosphere of 5% CO_2.

The co-cultured cells were harvested and $CD4^+$ T cells expression of CD28 and CD25 was analysed by FACS analysis of the resultant $CD4^+$ T cells from this population. Cells were stained (30 min at 4°C) with fluorochrome conjugated monoclonal antibodies Anti-Mouse CD3-FITC (clone KT3, Rat IgG2a), Anti-Mouse CD4-FITC (clone YTS, Rat IgG2b), Anti-Mouse CD4-PE (clone YTS, Rat IgG2b), Anti-Mouse CD8α-FITC (clone KT15, Rat IgG2a, λ), Anti-Mouse CD8α-PE (clone KT15, Rat IgG2a, λ), Anti-Mouse CD25-PE (clone Y6, Mouse IgM), Anti-Mouse CD28-PE (clone YTS, Rat IgG2b), (Antigenix America Inc., USA). For background fluorescence analysis isotype control monoclonal antibodies Anti-Rat IgG2a-FITC (clone YTS, Rat IgG2b) and Anti-Rat IgG2b-PE (clone YTS, Rat IgG2b) were used. The analysis was performed using Kaluza Flow Cytometry Analysis 1.0 software (Beckman Coulter Inc., Fullerton, CA, USA) (Supplementary Fig. S1, Supporting Information).

2.12 Study population

The serological assays were performed in a patient cohort comprising 85 female participans (33.5 ± 5.6 years) with atopy and a history of recurrent vaginal mycosis (Dept. Clin. Immunol. and Allergy). Inhalant allergy was present in 58 % of patients. The exclusion criteria were recent or ongoing antibiotic or immunosuppressive therapy. *Candida* spp., *Aspergillus* spp., and *Saccharomyces* spp. isolated from vaginal (91.53%) or cervical (6.38%) swabs undergone typing and identification (MEDIREX Inc., HPL Mycology Labs., Slovakia). Cultures were predominantely positive for *C. albicans* (82.27%). *C.glabrata* was identified in 7.26% and *S.cerevisiae* in 3.33%.

Alyostal® Stallergenes Skin prick test (SPT), including *C. albicans* allergen (Alyostal R Stallergenes), was performed on the patients' forearm according to the international and national guidelines. SPT was evaluated after 15–20 min and rated as positive if the wheal diameter was ≥3 mm and the negative control was negative.

2.13 Ethics

The research study and the protocol have been approved by the Ethical Committee of the Oncology Institute of St. Elisabeth, Bratislava, Slovakia (15.12.2010). Written informed consent to participate in the study and for blood collection and next laboratory examinations, in accordance with the principles in the Helsinki Declaration, was obtained from each patient prior to study enrolment. All patients were recruited from the outpatient department of the Department of Clinical Immunology and Allergy. Patient's age, disease process, drug history, family history, and clinical signs and symptoms were documented at the first visit of Clinical Immunology and Allergy ambulance as a standard procedure.

2.14 ELISA assay for specific anti–Candida albicans CCY 29-3-32,, C. glabrata CCY 26-20-1 and anti-Saccharomyces .cerevisiae CCY 21-4-13 mannan antibodies.

Sera samples have been taken ahead of the onset of antifungal and/or immunomodulative therapy, respectively. The sera samples for the determination of anti-mannan antibodies were separated and immediately stored at −70°C until the further use. The ELISA for the determination of specific anti-mannan IgG, IgA, and IgM isotypic antibodies based on *C. albicans, C. glabrata* and *S .cerevisiae* cell mannans has been developed by the modification of standard ELISA anti-*Candida* II and ELISA anti-ASCA (Biogema, Slovakia).

2.15 Statistical analysis

Data are reported as the means ± SD from three separate experiments. Statistical significance was determined using analysis of variance (ANOVA), corrected for multiple comparisons by the Bonferroni test. A value of $p < 0.05$ was considered significant.

3. **Results and Discussion**

3.1 Characterisation of C.glabrata mannan, chemical and structural features

For immunomodulatory properties analysis of *C. glabrata* mannan we prepared acido-stabile part of *C. glabrata* mannan by precipitation with Fehling's reagent. Prepared mannan with a molecular mass of 16.7 kDa contained no nitrogen (C, H, N analysis) and therefore this analysis confirmed that the mannan contained no protein. As published, the detailed mannan structures from different *C. glabrata* strains can differ and were correlated with antifungal drug susceptibility (Castanar*, et al.*, 2015).

Linkage types between mannose residues are determined from NMR. More specifically, they are determined from both proton and carbon chemical shifts in the anomeric region of hetero-correlated spectra. Acido-stable part of mannan from *C. glabrata* CCY 26-20-1 strain studied here does not contain any intense cross-peaks in the 4.5-4.9 ppm region for protons. That indicates absence of β glycosidic linkages. In the region above the 5 ppm, there are six intense cross-peaks (Fig. 1). Peaks at 5.06/103 ppm and 5.08/99 ppm are assigned to α-1,6 linked mannoses in the backbone - substituted or unsubstituted by side chains, respectively. There are intense signals at 5.28/101.3 ppm and 5.05/103 ppm and they are assigned to α-1,2 linked mannoses. The signals at 5.17/95 ppm and 5.15/103 ppm indicate the presence of α-1,3 linkages at the interior and terminal positions of side chains.

Here, we investigate mannan from *C. glabrata* 26-20-1 strain prepared by the method described in subsection 2.1. The TOCSY spectrum of the obtained mannan is in Fig. 2. The main structural features are following: Absence of any signal around 5.43 ppm and 5.56 ppm indicate that there is no phosphodiesterified structure. CP 15 at 4.78;4.05 ppm (in this spectrum invisible) and CP 6 at 5.18;4.28 ppm indicates a presence of a small portion of Manβ1→2Manα1→2 structure. Also, it has more (→6Manα1)$_n$→6Manα1→ (CP 4.89;3.94) and →6Manα1 (CP 8 at 5.11;4.04) units, and less Manα1→3Manα1→2 (CP 7 at 5.15;4.08 and CP 12 at 5.05;4.22) units than *S. cerevisiae* (21-4-13) mannan (see supplement for both spectra, Supplementary Fig. S2, Supporting Information).

The study of Grauss et al. (Graus*, et al.*, 2018) with mannosylation *C. albicans* and *C.glabrata* mutants revealed correlation between N-mannan structure and the amount of β-glucan exposed. They define specific N-mannan structural features of both yeasts and thei mutants affecting immune evasion via the alteration of glucan exposure geometries at the molecular level. Their findings pointed out the importancy N-mannan side-chain abundance and complexity in *C. albicans* and backbone length in *C. glabrata* as features that are important for masking glucan.

3.2 C. glabrata incidence.

C. glabrata is the second most common cause of systemic candidiasis in immuno- compromised patients following *C. albicans* (Yapar, 2014). Infections caused by *C. glabrata* accounts for approximately 18 - 29% of all bloodstream infection isolates in the USA (Pfaller*, et al.*, 2011, Pfaller*, et al.*, 2012, Yapar, 2014). In Slovakia, epidemiological data on yeast cultures obtained from patients displayed that *C. albicans* was the most frequently isolated species followed by *C. glabrata*. The main incidence of *C. glabrata* in yeast clinical isolates was localized in lower respiratory tract (2010-27%, 2013-31.9%, 2016-25.16%) followed by upper respiratory tract (2010 - 22%, 2013 - 16.6%, 2016 - 16.2%) (Fig. 3). Decreased susceptibility of *C. glabrata* to azoles is eminent (Pfaller*, et al.*, 2012) and the emergence of co-resistance to the echinocandins among fluconazole-resistant *C. glabrata* isolates was documented (Pfaller*, et al.*, 2012).

3.3 Distribution of antimycotic antibodies in vulvovaginitis patients

The serologic analysis of anti-mannan antibodies in cohort of atopic females with episodes of mycotic colpitis revealed that highest concentrations of antifungal antibodies are of IgM isotype, followed by IgG class antibodies.The majority of positive results over normal reference interval is observed with IgM isotype antibodies against*, C.albicans* and *C. glabarata*, less in the case of *S. cerevisiae* mannan (Tab.1).

Generally, IgM antibodies represent the most abundant immunoglobulin isotype, involved in early infection. IgG antibodies are considered as indicator of past or ongoing infection, elevated IgG is characteristic for reccurent attacs (Casadevall, 1995). Our previous results on serological pattern of specific antibodies anti-synthetically prepared galactomannosides mimicking the structure of natural *Aspergillus* cell revealed the highest levels also with antigen-specific IgM isotypic antibodies glycan in mycotic colpitis cohort (Paulovicova*, et al.*, 2017). Recently, the role of natural IgM antibodies as scavenger, protector and regulator is stressed (Ehrenstein & Notley, 2010, Gronwall*, et al.*, 2012).

3.4 Splenocytes

3.4.1 Proliferation of mannan exposed splenocytes

We examined the ability of isolated *C. glabrata* mannan to induce proliferative response of splenocytes and we compared obtained results with effectiveness of LPS (Fig. 4). *C. glabrata* mannan slightly increased the proliferation of splenocytes. Both concentrations used for stimulation (400 µg/ml and 800 µg/ml) induced comparable statistically significant increases (1.29 times higher resp. 1.25 times higher compared to unstimulated splenocytes) of splenocytes proliferation (Fig. 4). Despite published negative influence of mannan isolated by Fehling method onto proliferation of lymphocytes (Nelson*, et al.*, 1991) we observed increase of splenocytes´ proliferation induced by *C. glabrata* mannan.

3.4.2 Mannan induced splenic proinflammatory cytokines

Given that cytokines could act not only as modulators of antifungal effector functions but also as important mediators of the regulation of Th lymphocyte repertoire development (Romani, 1999) we tested whether *C. glabrata* mannan stimulation have the ability to induce cytokines production of splenocytes.

We analysed the TNF-α production of *C. glabrata* mannan stimulated splenocytes and obtained results compared with unstimulated and LPS stimulated splenocytes by ELISpot method (Fig. 5). *C. glabrata* mannan induced significant increase of TNF-α producing cells frequency, even comparable to the efficacy of LPS as positive control. Interestingly, the lower concentration of *C. glabrata* mannan (400 µg/ml) induced slightly higher number of TNF-α producing cells in comparison with higher concentration (800 µg/ml).

As expected, according to results of TNF-α producing cells frequency, stimulation of splenocytes with *C. glabrata* mannan induced significant increase of TNF-α concentration in culture media, 400

μg/ml of mannan 13.8 times higher and 800 μg/ml of mannan 41.0 times higher in comparison with unstimulated splenocytes (Fig. 6). Stimulation of splenocytes with mannan did not significantly influence the production of IL-6 and IFN-γ, but increased the production of IL-4. Statistically significantly higher IL-4 concentration (1.6 times higher compared to unstimulated control) was detected by using 400 μg/ml of *C. glabrata* mannan for stimulation (Fig. 6). The lower concentration of mannan was more effective in evocation of IL-4 production than the higher concentration. These results reveal inversely proportional dependence between IL-4 induction upon stimulation of splenocytes and used mannan concentration.

Previous studies indicated that a mannan with a highly branched structure exhibited stronger pyrogenic activity than less branched mannans and the TNF-α-inducing ability of mannan was also affected by proportions and length of the branches of α-(1-2)- and α-(1-3)-linked mannopyranosyl residues (Tada*, et al.*, 2002). *S. cerevisiae* mannan showed higher activity than *C. albicans* mannan and therefore variation of structure, especially the proportion of branching in different mannan preparations, might be responsible for variation of cytokine-inducing activities (Tada*, et al.*, 2002).

3.5. RAW 264.7 cell line

3.5.1 Mannan-induced RAW264.7 proliferation

Next, we compared the proliferative response of RAW 264.7 macrophages (Fig. 7) to *C. glabrata* mannan stimulation with mannan *C. albicans* mannan and *S. cerevisiae* mannan at different concentrations (100, 300, 400, 600 and 800μg/ml). We observed that all three used mannans were able to stimulate proliferation of RAW 264.7 macrophages, especially after 48 h treatment. Obtained results showed slight differences in used mannans capability to induce proliferation (concentration dependent effect, Fig. 7). It was described, that mannan-derived oligosaccharides produced by catabolism of mannan *in vivo* are immunoinhibitory and contribute to the deficit in cell-mediated immune function associated with chronic candidiasis (Podzorski*, et al.*, 1990).

3.5.2 Mannan-induced RAW264.7 macrophages'cytokine release

To analyse differences of *C. glabrata* mannan, *C. albicans* mannan and *S. cerevisiae* mannan capability to induce cytokines production, RAW 264.7 macrophages were treated with five concentrations of each mannan (100, 300, 400, 600 and 800μg/ml) and subsequently culture media were analysed (Fig. 8).

Obtained results reveal significant differences in the induction of TNFα production, the highest for *C. albicans* mannan, lower for *S. cerevisiae* mannan and the lowest for *C. glabrata* mannan. The IL-6 concentration was statistically significantly increased only after *C. albicans* mannan stimulation. Also IL-12(p70) levels in culture media after *C. albicans* mannan treatment of RAW 264.7 macrophages most markedly statistically significantly increased. The *S. cerevisiae* mannan slightly increased the IL-12(p70) production and *C. glabrata* mannan stimulation of macrophages for 48 h induced even decrease of IL-12(p70) production, especially the highest concentration (800µg/ml, 0.49 times lower than control). The *C. glabrata* mannan and *S. cerevisiae* mannan did not significantly alter the IL-10 production, but *C. albicans* mannan significantly increased the IL-10 levels. Obtained results concerning *C. glabrata* mannan stimulatory activity did not reveal concentration dependent manner. This mainly concentration dependent activity was observed for *C. albicans* mannan and *S. cerevisiae* mannan, with higher efficacy for *C. albicans* mannan (Fig. 8).

Previously published results of inflammatory cytokine production induced by mannan lacking β-1,2-mannosides (Ueno*, et al.*, 2013) shoved concentration dependent cytokine release in the *C. albicans* mannan concentration range 4-100 µg/ml. They observed, that β-1,2-mannosides mitigated the production by mouse DCs of inflammatory cytokines, including IL-6, IL-12p40 and TNF- α (Ueno*, et al.*, 2013). Our results, obtained for *C. glabrata* mannan and *C. albicans* mannan did not confirmed this assumption.

Candida mannan is recognized by various specific receptors of immune cellse.g. mannose receptor, complement receptor 3, dectin-2, DC-SIGN, TLR- 2, TLR-4 and TLR-6 (Netea*, et al.*, 2008, Ueno*, et al.*, 2013) depending on its structure (Romani, 2011, Hall & Gow, 2013). The mannose receptor (MR, also known as CD206), a transmembrane lectin found predominantly in macrophages can recognize terminal mannose structures (Netea*, et al.*, 2008, Vautier*, et al.*, 2012).

RAW264.7 macrophages, expressing both dectin-2 and dectin-3, produced greater amounts of TNF-α_ in response to purified _α-mannans compared to the cells expressing only one of these receptors (Zhu*, et al.*, 2013). O-linked mannans on the surface of *C. albicans* yeast cells are recognised by TLR4, expressed on both human mononuclear cells and murine macrophages. The interaction of MR and TLR4 with N- and O-linked mannans, respectively trigerred induced release of TNF_, IL-6, IL-10, and IFNγ by these cells(Netea*, et al.*, 2006). Chen et al (Chen*, et al.*, 2017) using a macrophage-*C. glabrata* interaction model revealed, that *C. glabrata* could activate NF-kB signaling, which including nuclear translocation of NFkB (p65), Syk phosphorylation, IkBa phosphorylation, together with IkBa degradation in thioglycolate-elicited peritoneal macrophages. Subsequently, they observed *C. glabrata* (UV-inactivated and live *C. glabrata)* induced release of T_H polarizing cytokines

including pro- inflammatory cytokines, TNF-a, IL-6, IL-12p40, and antiinflammatory IL-10, in thioglycolate-elicited peritoneal macrophages.

3.5 Dentritic cells

3.5.1 Mannan-induced expression of activation and co-stimulatory antigens in dendritic cells

Antigen presentation and immune recognition are two critical events in the generation of effective immune responses to microbial pathogens. Initiation and maintenance of specific T cell responses rely on the interaction of the fungus with professional antigen-presenting cells (APCs). DCs are efficient in priming and expanding *Candida* specific T lymphocytes in the inductive sites of the immune system (Romagnoli*, et al.*, 2004). The first signal is the occupancy of the T-cell receptor (TCR) by a complex of the antigenic peptide and major histocompatibility complex (MHC) molecules on the APC surface. The second signal results from binding co-stimulatory ligand molecule on the APC surface to a receptor on the T-cell surface. The major co-stimulatory signal appears to be provided by the B7 molecules, B7-1 (CD80) and B7-2 (CD86), on the DCs. CD80 and CD86 are ligands for the T-cell membrane proteins CD28 and CTLA-4 and have been concerned as important determinants on professional APCs that play a major role in $CD4^+$ T cell activation (Zhang*, et al.*, 2004).

We analyzed the capacity of purified *C. glabrata* mannan to induce the activation of splenic DCs. For optimization of the DCs treatment and to set the optimal time period for the highest expression of activation antigens CD80 and CD86 on DCs we performed stimulation with one concentration of *C. glabrata* mannan (400 μg/ml) and as a positive control for stimulation we used *Escherichia coli* LPS (10 μg/ml) (Fig. 9). Results reveal 24 h as optimal time for induction of CD80 and CD86 expression on DCs. After 48 h stimulation, we observed decrease in CD80 and CD86 expression compared to 24 h treatment (Fig. 9). According to obtained results, we used 24 h treatment of DCs for all following experiments.

Based on the concentration-dependent stimulatory efficacy of mannan, we analysed the activity of various concentrations of *C. glabrata* mannan (100 μg/ml, 300 μg/ml, 600 μg/ml) onto activation of purified DCs (Fig. 10).

The cells were analyzed for CD80 and CD86 expression. The reported results (Fig. 10) indicate that all three tested concentrations of mannan *C. glabrata* are able to induce increase of CD80 and CD86 expression on DCs. When compared different stimulant concentration, we observed, that after 24 h stimulation with concentration of 100 μg/ml of mannan *C. glabrata* the significant increase of both co-stimulatory molecules expression was induced (Fig. 10). Splenic DCs stimulated with 300 μg/ml of mannan retain high expression of CD80 but we observed decrease of CD86 expression.

Stimulation of DCs with the highest tested concentration of mannan (600 μg/ml) significantly decreased the efficacy of mannan to induce expression of CD80 on DCs. By using mannan concentration 600 μg/ml, the expression of CD86 significantly decreased (Fig. 10).

To evaluate DCs and T lymphocytes co-stimulation we analysed the expression of CD28 on $CD4^+$ T cells after co-cultivation with *C. glabrata* mannan pulsed DCs (Fig. 11). For the determination of $CD4^+CD28^+$ T cells induction we used the concentration 100 μg/ml of *C. glabrata* mannan, based on the highest induction of B7 co-stimulatory molecules (CD80, CD86) on DCs. After 48 hours of co-cultivation, we observed slight increase of CD28 on $CD4^+$ T cells (1.4 times higher than unstimulated control). Extended duration of mannan pulsed DCs and lymphocytes co-cultivation (120 hours) induced an additional increase of $CD4^+CD28^+$ T cells proportion within the $CD4^+$ T cells (Fig. 11). After 120 hours of co-cultivation $CD4^+CD28^+$ T cells the percentage was significantly higher (1.5 times) compared to unstimulated control and even comparable with LPS pulsed DCs as a positive control (Fig. 11). The activation of $CD4^+$ T cells *in vitro* was monitored by expression of CD25, an alpha chain of the IL-2 receptor (Fig. 11). We observed slight increase of $CD4^+CD25^+$ T cells proportion within the $CD4^+$ T cells upon co-stimulation with mannan pulsed DCs (both, 48 hours and 120 hours of co-cultivation) compared to unstimulated cells (Fig. 11).

In vitro and *in vivo* models have demonstrated the importance of both co-stimulations in the generation of antigen-specific immune responses (Zhang*, et al.*, 2004). It was shown that both ligands have a critical role in the activation of $CD4^+$ T cells *in vitro* and *in vivo* (Zhang*, et al.*, 2004). It has been suggested that the relative expression of CD80 and CD86 on APCs may have different functional consequences, such as driving T-cell differentiation into either the Th1 or the Th2 pathway. Both CD80 and CD86 could have distinct effects on the generation of T cell responses to different antigens and can compensate for each other in T cell activation and proliferation (Zhang*, et al.*, 2004). In this study, we analyse the interaction of mannan from *C. glabrata* with dendritic cells and demonstrate that mannan stimulates DCs and induces DCs maturation by increasing costimulatory molecules CD80 and CD86. Concentration dependent induction of CD80 and CD86 expression reveals differences in mannan ability to increase expression of costimulatory molecules on DCs.

4. Conclusions

Reported results provide new information about the possible role of the mannan antigen in the induction of immune response and show that mannan could be a useful tool to promote DCs

maturation and activation and, as a consequence, antigen presentation process. DCs are potential vehicles to induce or potentiate protective immune response and prevent fungal infection.

The model of DCs is a promising target for immunotherapy interventions and vaccine development. The most opportunistic fungal pathogens need a stable host– microbe interaction. The aim of this study was to evaluate capability of mannan to modulate DCs activation, induce expression of co-stimulatory molecules CD80, and CD86 on DCs as well as antigen presentation activity, thereby influencing T cell phenotype in response to stimulation. This study proved applicability of low molecular mannan as efficient molecular immunomodulator which was used in our consequent study for binding onto nanoliposomes via orthogonal aminooxy ligation (Bartheldyová*, et al.*, In Press). Moreover, nanoliposomes with orthogonally bound mannan represent a platform for development of targeted drug delivery systems and self-adjuvanted carriers for construction of recombinant vaccines.

Conflict of interest

The authors declare that there are no conflicts of interest.

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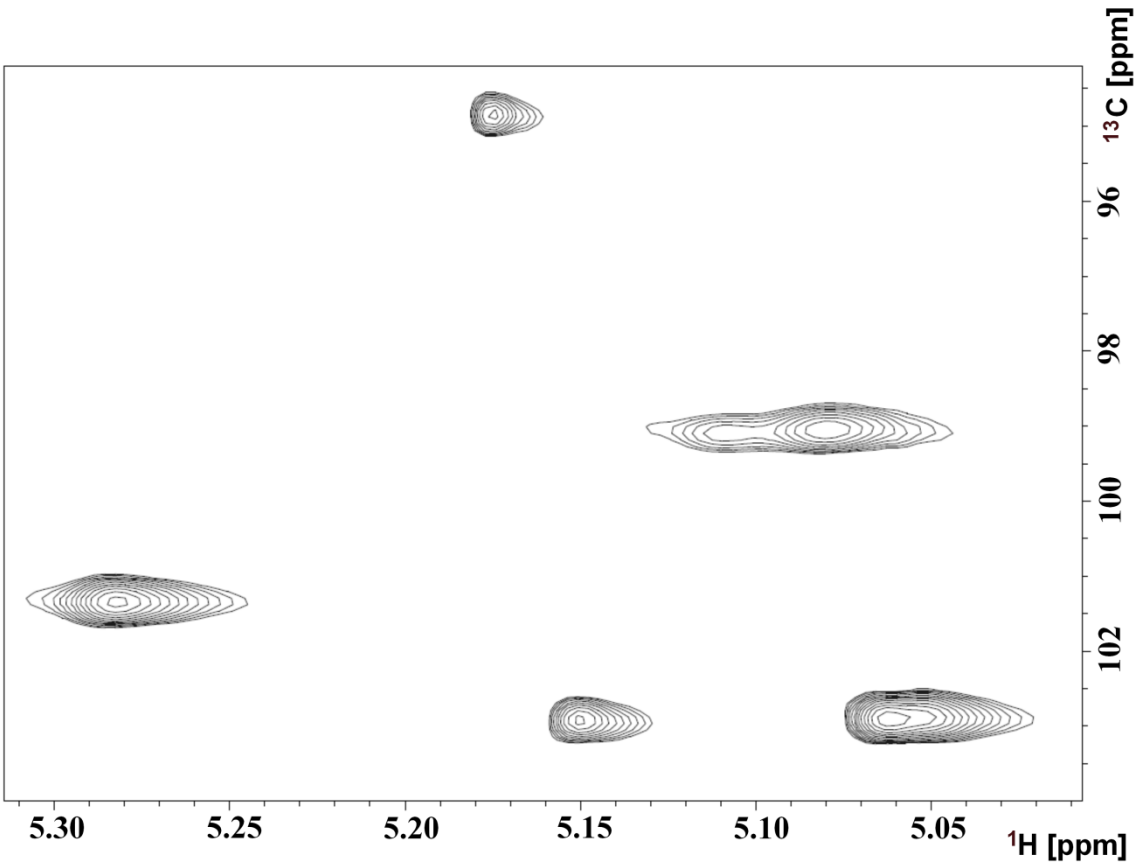

Fig. 1: Anomeric region of HSQC-NMR spectra of acido-stable part of mannan from *C. glabrata* CCY 26-20-1 measured at 45°C in D_2O. Aceton was used as internal standard (2.225/31.07 ppm).

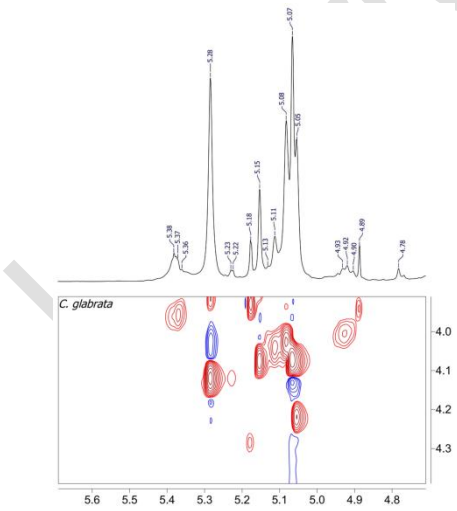

Fig. 2: TOCSY NMR spectrum of the mannan, detail on the anomeric part (D_2O, 45 °C, 600 MHz). The numbering of cross-peaks is according to Takahashi et al. (Takahashi*, et al.*, 2012).

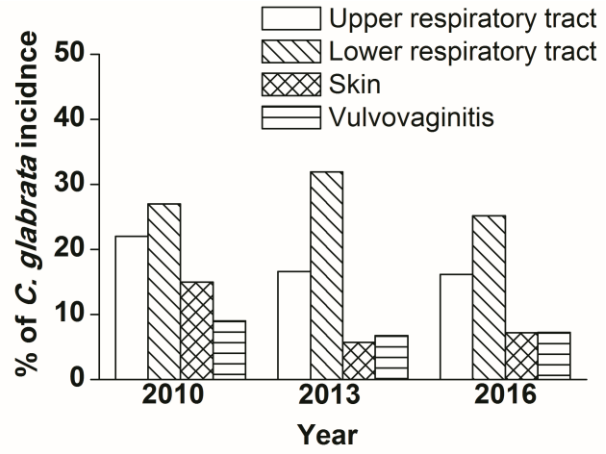

Fig. 3: Incidence of *C. glabrata* in yeast clinical isolates (years 2010, 2013, and 2016) according to location of infection (with permission of MEDIREX Inc., HPL Mycology Labs., Slovakia)

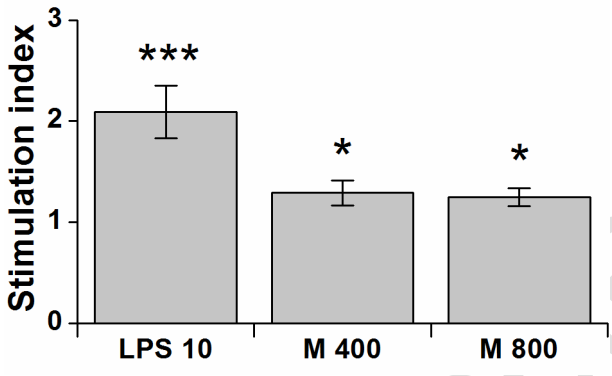

Fig. 4: Proliferation of splenocytes

Splenocytes were stimulated for 24 h with *E. coli* LPS (LPS, 10µg/ml) and mannan *C. glabrata* (M400 - 400µg/ml, M800 - 800µg/ml). As a negative control, splenocytes cultured without stimulants were used. Results are expressed as a mean stimulation indexes (average relative light units in the presence of antigens/average relative light units obtained without antigen). All data are presented as a mean stimulation indexes ± SD and statistical significances of difference between unstimulated cells (negative control, stimulation index 1) and stimulated cells are expressed: *** - $P<0.001$, * - $0.01<P<0.05$.

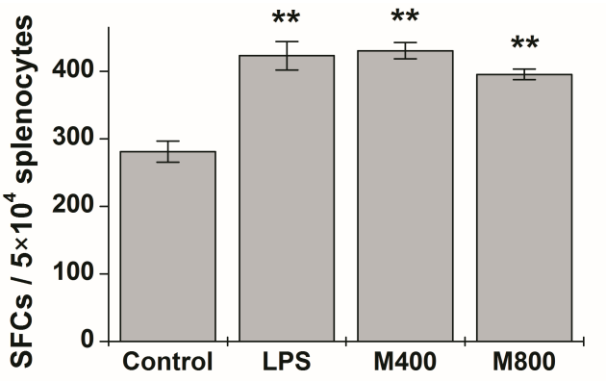

Fig. 5: Splenocytes production of TNF-α Splenocytes were stimulated for 24 h with *E. coli* LPS (LPS, 10µg/ml) and mannan *C. glabrata* (M400 - 400µg/ml, M800 - 800µg/ml). As a negative control, splenocytes cultured without stimulants (Control) were used. All data are presented as a mean frequency of spot forming cells (SFCs) per 5×10^4 splenocytes ± SD and statistical significance of differences between unstimulated cells (Control) and stimulated cells are expressed: ** - $0.001<P<0.01$, * - $0.01<P<0.05$.

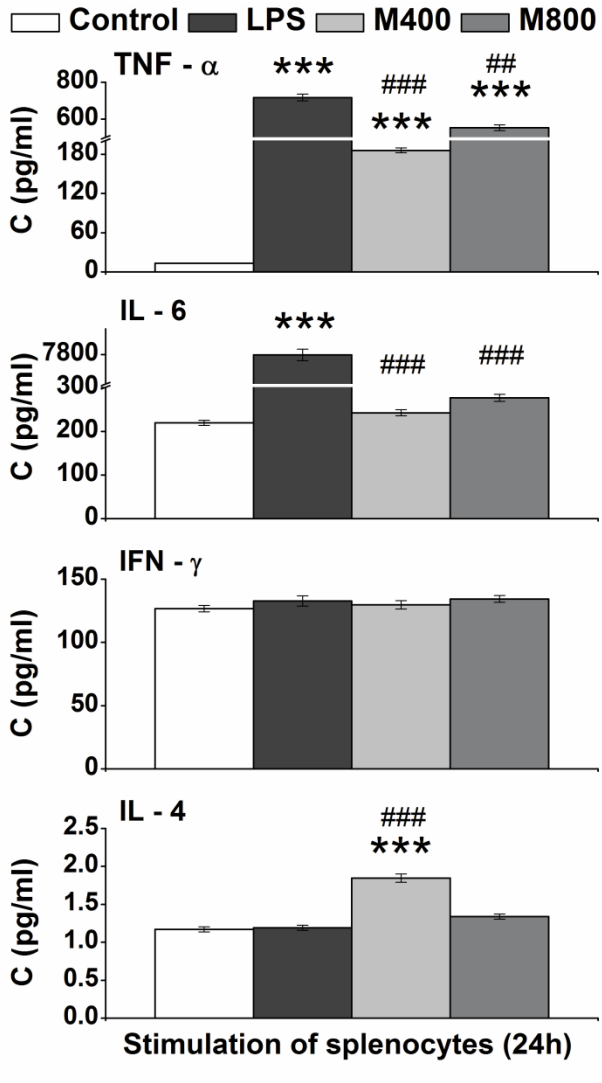

Fig. 6: Cytokines´pattern induced by mannan and LPS

Culture media after splenocytes stimulation were analysed for cytokines concentration. Splenocytes were stimulated for 24 h with *E. coli* LPS (LPS, 10μg/ml) and mannan *C. glabrata* (M400 – 400 μg/ml, M800 – 800 μg/ml). As a negative control, splenocytes cultured without stimulants (Control) were used. All data are presented as mean ± SD. Statistical significance of differences between stimulated cells and unstimulated cells (Control) are expressed (*** - P<0.001, ** - 0.001<P<0.01, * - 0.01<P<0.05) and statistical significance of differences between LPS stimulated cells and mannan stimulated cells are expressed (### - P<0.001, ## - 0.001<P<0.01).

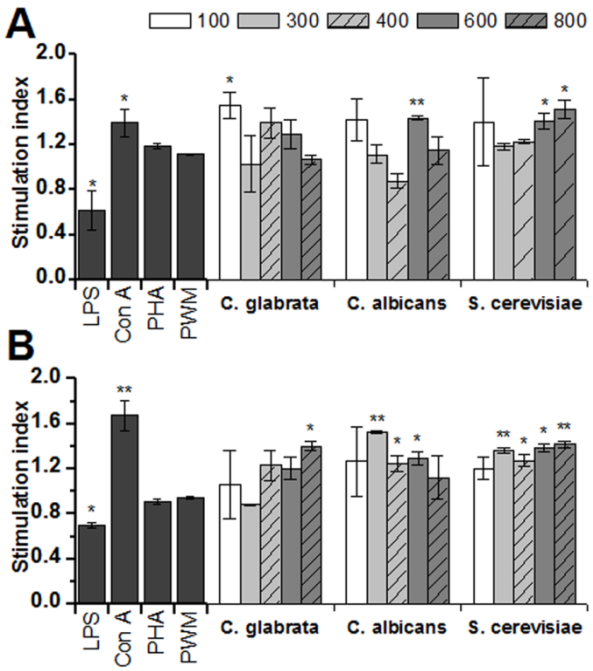

Fig. 7: Proliferation of RAW 264.7 macrophages

RAW 264.7 cells were stimulated for 24 h (A) and 48 h (B) with Con A (10 µg/mL), PHA (10 µg/mL), PWM (1 µg/ mL), and LPS (10 µg/mL) were used as positive controls and *C. glabrata* mannan, *C. albicans* mannan and *S. cerevisiae* mannan (100, 300, 400, 600 and 800µg/ml). As a negative control, RAW 264.7 cells cultured without stimulants were used. Results were calculated as a stimulation indexes (average relative light units in the presence of antigens/average relative light units obtained without antigen). All data are presented as a mean stimulation indexes ± SD and statistical significances of difference between unstimulated cells (negative control, stimulation index 1) and stimulated cells are expressed: ** - 0.001<P<0.01, * - 0.01<P<0.05.

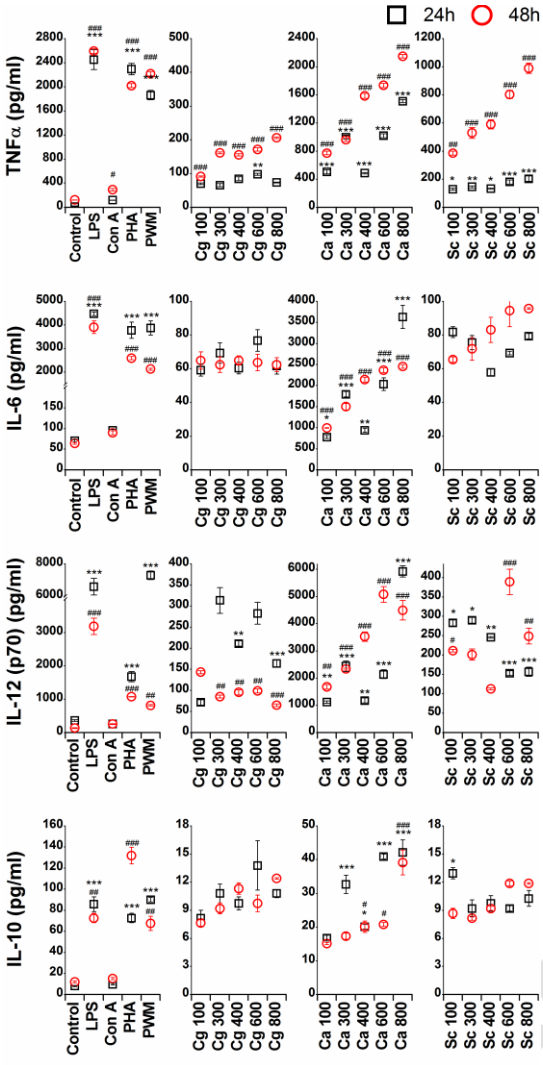

Fig. 8: RAW264.7 macrophages'cytokine release induced by mannan

Culture media after stimulation of RAW 264.7 macrophages were analysed for cytokines concentration. Cells were stimulated for 24 and 48 h with Con A (10 µg/mL), PHA (10 µg/mL), PWM (1 µg/ mL), and LPS (10 µg/mL), used as positive controls, and *C. glabrata* mannan (Cg), *C. albicans* mannan (Ca) and *S. cerevisiae* mannan (Sc) (100, 300, 400, 600 and 800µg/ml). As a negative control (Control), RAW 264.7 cells cultured without stimulants were used. All data are presented as mean ± SD. Statistical significances of difference between stimulated cells and unstimulated cells (Control) after 24 h treatment are expressed (*** - P<0.001, ** - 0.001<P<0.01, * - 0.01<P<0.05) and after 48 h treatment are expressed (### - P<0.001, ## - 0.001<P<0.01, # - 0.01<P<0.05).

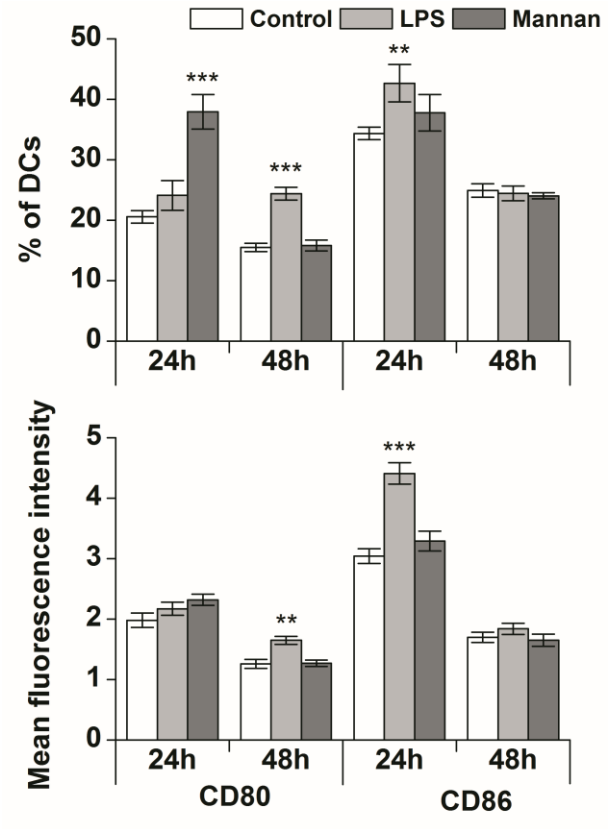

Fig. 9: Expression of CD80 and CD86 upon DCs stimulation

DCs were stimulated for 24 h and 48 h with 10 µg/ml *E. coli* lipopolysaccharide (LPS) and 100 µg/ml of mannan *C. glabrata* (Mannan). DCs cultured without stimulants (Control) were used as a negative control. Data are expressed as mean percentage of $CD80^+$ DCs and mean percentage of $CD86^+$ DCs out of $CD11c^+$ DCs ± SD and the mean fluorescence intensity of $CD80^+$ DCs and $CD86^+$ DCs.

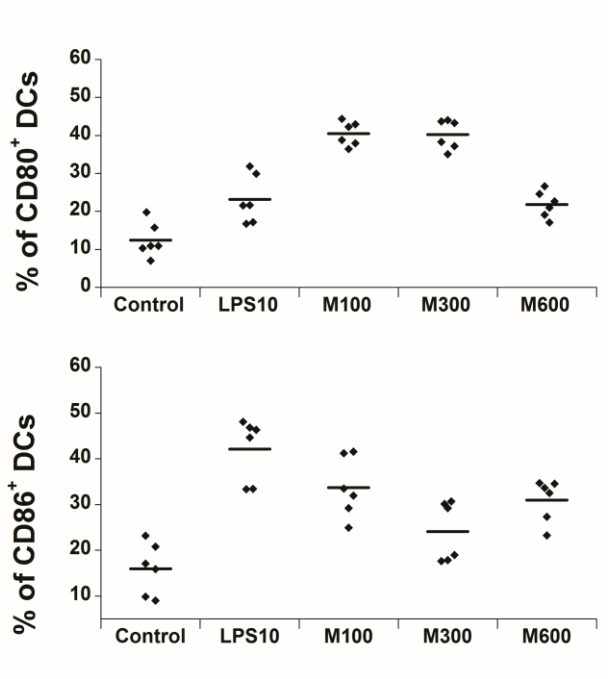

Fig. 10: Expression of CD80 and CD86

DCs were stimulated for 24 h with 10 µg/ml *E. coli* lipopolysaccharide (LPS10), 100 µg/ml mannan *C. glabrata* (M100), 300 µg/ml mannan *C. glabrata* (M300) and 600 µg/ml mannan *C. glabrata* (M600).

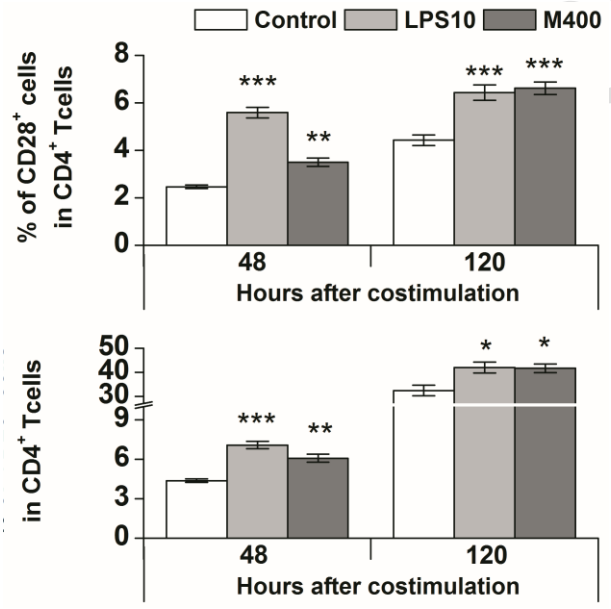

Fig. 11: Expression of CD28 and CD25 on $CD4^+$ T lymphocytes

DCs were stimulated for 24 h with 10 µg/ml *E. coli* lipopolysaccharide (LPS10), 100 µg/ml mannan *C. glabrata* (M100). Antigen pulsed DCs were co-cultured with non-adherent cells obtained after $CD11c^+$ DCs isolation for 48 hours and 120 hours.

Table 1. Serologic profiles of anti-*Candida albicans*, anti-*Candida glabrata* and anti-*Saccharomyces cerevisiae* isotypic antibodies.

	Anti-*Candida albicans*	**Anti-*Candida glabrata***	**Anti-*Saccharomyces cerevisiae***
IgG (UmL^{-1})			
average ± s.d.	**45.179 ± 68.452**	**81.498 ± 131.734**	**36.788 ± 47.92**
positive results (%)	**36**	**53.8**	**33**
IgM (UmL^{-1})			
average ± s.d.	**216 ± 458.343**	**142.01 ± 107.368**	**57.279 ± 47.386**
positive results (%**)**	**70**	**79.5**	**60**
IgA (UmL^{-1})			
average ± s.d.	**39.596 ± 31.903**	**33.939 ± 33.365**	**24.097 ± 24.275**
positive results (%)	**20**	**30.7**	**43**